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(54) Title: FLUORESCENT MULTIPLEX HPV PCR ASSAYS USING MULTIPLE FLUOROPHORES

(57) Abstract: The present invention relates a fluorescent multiplex PCR assay for detecting the presence of an HPV subtype in a sample using multiple fluorophores to simultaneously detect a plurality of HPV genes of the same HPV subtype. The present invention also relates to primer pairs and probes specific to HPV subtypes for use in the methods of the present invention.

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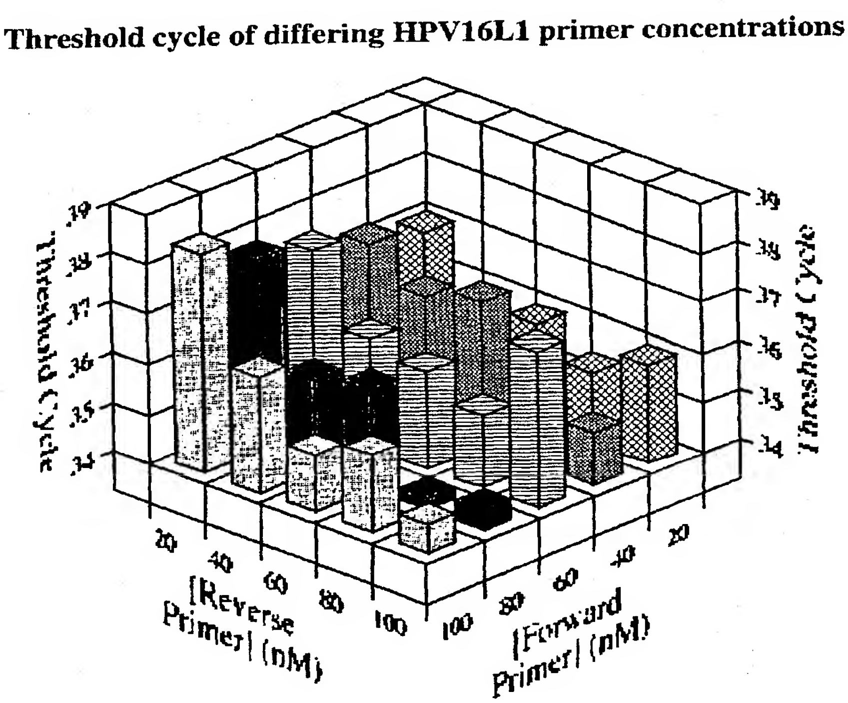

(57) Abstract: The present invention relates a fluorescent multiplex PCR assay for detecting the presence of an HPV subtype in a sample using multiple fluorophores to simultaneously detect a plurality of HPV genes of the same HPV subtype. The present invention also relates to primer pairs and probes specific to HPV subtypes for use in the methods of the present invention.

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TITLE OF THE INVENTION

FLUORESCENT MULTIPLEX HPV PCR ASSAYS USING MULTIPLE FLUOROPHORES

FIELD OF THE INVENTION

The present invention relates generally to PCR-based assays to detect the presence of human papillomavirus (HPV) subtypes in clinical samples. More specifically, it relates to a fluorescent multiplex PCR assay, wherein multiple fluorophores are used to simultaneously detect a plurality of HPV loci in a single PCR reaction tube.

BACKGROUND OF THE INVENTION

There are more than 80 types of human papillomavirus (HPV) that cause a wide variety of biological phenotypes, from benign proliferative warts to malignant carcinomas (for review, see McMurray et al., *Int. J. Exp. Pathol.* 82(1): 15-33 (2001)). HPV6 and HPV11 are the types most commonly associated with benign warts, whereas HPV16 and HPV18 are the high-risk types most frequently associated with malignant lesions. Determination of the specific type of HPV in a clinical sample is, therefore, critical for predicting risk of developing HPV-associated disease.

Several nucleic acid-based methods have been utilized to identify and quantify specific HPV types in clinical samples, such as detection of viral nucleic acid by in situ hybridization, Southern blot analysis, hybrid capture or polymerase chain reaction (PCR). PCR-based methods often involve amplification of a single specific HPV target sequence followed by blotting the resulting amplicon to a membrane and probing with a radioactively labeled oligonucleotide probe.

Other methods exploit the high homology between specific HPV genes of different subtypes through the use of commercially available consensus primers capable of PCR amplifying numerous HPV subtypes present in a sample. The presence of a specific HPV subtype is then identified using a subtype-specific oligonucleotide probe. See, e.g., Kleter et al., *Journal of Clinical Microbiology* 37(8): 2508-2517 (1999); Gravitt et al., *Journal of Clinical Microbiology* 38(1): 357-361 (2000). Similarly, assays that utilize degenerate PCR primers take advantage of the homology between HPV subtypes, allowing detection of a greater number of HPV types than methods utilizing specific primer sets. See, e.g. Harwood et al., *Journal of*

Tucker et al. (*Molecular Diagnosis* 6(1): 39-47 (2001)) describe an assay that targets a conserved region spanning the E6/E7 junction. Like the Josefsson assay, Tucker et al. employed real-time detection and type-specific fluorescent probes. Tucker et al. also utilized multiplex PCR to simultaneously detect HPV target sequences and either the actin or globin cellular loci in the same reaction tube.

The methods described above typically involve testing for the presence of a single viral locus in a DNA sample such as the L1 locus. A disadvantage of single-locus assays is that the high degree of homology among specific HPV genes from one HPV type to another leads to an excessive occurrence of false positive results. This level of homology makes it difficult to design a PCR assay that is specific for a single HPV type. It is therefore necessary to confirm positive results by testing for the presence of several loci of a single HPV-type. The further experimentation required to verify positive results is cumbersome and time-consuming. Establishment of the HPV status of a clinical sample for four different HPV types typically consumes 26-30 man-hours.

Single-locus assays may also lead to false negative results. It is well established that the relationship between the HPV genome and chromosomal host DNA may change during the multistage tumorigenic process (For review, see McMurray et al., *Int. J. Exp. Path.* 82: 15-33 (2001)). Premalignant lesions are often associated with episomal forms of HPV DNA while later-stage tumors typically have integrated HPV sequences. As a result of the integration correlated with advanced stages of disease progression, the open reading frame of specific HPV genes, such as the L1 gene, may become disrupted. Such disruption of HPV gene sequences may lead to false negative results in assays that target the disrupted sequence.

Despite the development of the HPV assays described above, it would be advantageous to develop an assay that is highly sensitive and reproducible, and that requires reduced man-hours compared to methods disclosed in the art.

SUMMARY OF THE INVENTION

The present invention relates to a fluorescent multiplex PCR assay for detecting the presence of an HPV subtype in a sample which uses multiple fluorophores to simultaneously detect a plurality of HPV loci of the same HPV subtype.

example, a forward discriminatory primer hybridizes to the E6 gene and a reverse discriminatory primer hybridizes to the E7 gene. As a result, at least one PCR amplicon comprises a sequence of nucleotides derived from more than one gene. The oligonucleotide probe specific to said amplicon may hybridize, for example, to a sequence of nucleotides derived from the E6 gene, a sequence of nucleotides derived from the E7 gene, or a sequence of nucleotides that crosses the E6/E7 boundary.

In a preferred embodiment of this invention, the HPV subtype is selected from the group consisting of: HPV6, HPV11, HPV16 and HPV18.

In a further preferred embodiment of the method of the present invention, the number of oligonucleotide sets is three and the oligonucleotide sets specifically hybridize to the E6, E7 and L1 genes of HPV. A sample is positive for the HPV subtype being tested if two or three of the E6, E7 or L1 genes are detected.

Another embodiment of this invention relates to an oligonucleotide probe comprising a sequence of nucleotides specific to a single HPV type. Said oligonucleotide probe can bind to specific HPV amplicons resulting from PCR amplification of viral DNA using specific oligonucleotide primers. In a further embodiment of this invention, said oligonucleotide probe comprises a sequence of nucleotides selected from the group consisting of: SEQ ID NO:3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:18, SEQ ID NO:21, SEQ ID NO:24, SEQ ID NO:27, SEQ ID NO: 30, SEQ ID NO: 33 and SEQ ID NO:36.

The present invention also relates to said oligonucleotide probes further comprising a fluorophore and a quencher molecule. In a preferred embodiment of this invention, the fluorophore is selected from the group consisting of: FAM, JOE and TET and the quencher is non-fluorescent. In an especially preferred embodiment of this invention, the quencher is BHQ1.

The present invention further relates to a primer pair for the PCR amplification of HPV nucleic acid, wherein both the forward and reverse PCR primers are discriminatory. In a preferred embodiment of the invention, the nucleotide sequences of the primer pair are selected from the group consisting of: SEQ ID NO:1 and SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5, SEQ ID NO:7 and SEQ ID NO:8, SEQ ID NO:10 and SEQ ID NO:11, SEQ ID NO:13 and SEQ ID NO:14, SEQ ID NO:16 and SEQ ID NO:17, SEQ ID NO:19 and SEQ ID NO:20, SEQ ID NO:22 and SEQ ID NO:23, SEQ ID NO:25 and SEQ ID NO:26, SEQ ID NO:28

As used herein, the term "discriminatory," used in reference to the oligonucleotide primers and probes of the present invention, means that said primers and probes are specific to a single HPV subtype. It includes HPV primers and probes specific to a single HPV subtype, but that share some homology with other HPV subtypes. "Discriminatory" primers and probes of the present invention include those oligonucleotides that lack 3' homology with other HPV subtypes in at least one nucleotide or more. Such a residue that is unique for the specific HPV subtype at the specific position and acts to discriminate the HPV subtype from the others in the alignment referred to as a "discriminatory base". The term "discriminatory," in reference to oligonucleotides, does not include primers and probes that are specific to more than one HPV subtype, i.e. those that share full homology with greater than one HPV subtype.

As used herein, "amplicon" refers to a specific product of a PCR reaction, which is produced by PCR amplification of a sample comprising nucleic acid in the presence of a nucleic acid polymerase and a specific primer pair. An amplicon can consist of a nucleotide sequence derived from a single gene of a single HPV subtype or an amplicon can consist of a nucleotide sequence derived from more than one gene of a single HPV subtype.

As used herein, "oligonucleotide set" refers to a grouping of a pair of oligonucleotide primers and an oligonucleotide probe that hybridize to a specific nucleotide sequence of a single HPV subtype. Said oligonucleotide set consists of: (a) a forward discriminatory primer that hybridizes to a first location of an HPV subtype; (b) a reverse discriminatory primer that hybridizes to a second location of the HPV subtype downstream of the first location and (c) a fluorescent probe labeled with a fluorophore and a quencher, which hybridizes to a location of the HPV subtype between the primers. In other words, an oligonucleotide set consists of a set of specific PCR primers capable of initiating synthesis of an amplicon specific to a single HPV subtype, and a fluorescent probe which hybridizes to the amplicon.

As used herein, "plurality" means two or more.

As used herein, "specifically hybridizes," in reference to oligonucleotide sets, oligonucleotide primers or oligonucleotide probes, means that said oligonucleotide sets, primers or probes hybridize to a single HPV subtype.

As used herein, "gene" means a segment of nucleic acid involved in producing a polypeptide chain. It includes both translated sequences (coding region) and 5' and 3' untranslated sequences (non-coding regions) as well as intervening

As used herein, "FAM" refers to the fluorophore 6-carboxy-fluorescein.

As used herein "JOE" refers to the fluorophore 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein.

As used herein, "TET" refers to the fluorophore 5-tetrachloro-fluorescein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the sequence of the oligonucleotide primers and probes used in the TaqMan multiplex PCR reactions, their positions within the open reading frame (A of start codon = 1), and the final concentration of each oligo in the multiplex PCR reaction. The six primers for each HPV type were combined into one concentrated (100X) hexa-primer stock. Each probe was stored as a concentrated (100X) stock.

FIGURE 2 shows a HPV16L1-FAM TaqMan dual-labeled probe concentration curve. The mean (n=3) threshold cycle ± SD was determined for escalating HPV16L1 probe concentrations in a 50 µl TaqMan PCR reaction using 100 copies of HPV16L1 plasmid as template DNA. Columns with the same letter are not significantly different ($p>0.05$) based on one-way ANOVA and Tukey's multiple comparison post-test analyses.

FIGURE 3 shows a HPV16L1-FAM TaqMan dual-labeled probe concentration curve. The mean (n=3) ΔRn (Rn-baseline) ± SD was determined for escalating HPV16L1 probe concentrations in a 50 µl TaqMan PCR reaction using 100 copies of HPV16L1 plasmid as template DNA. Columns with the same letter are not significantly different ($p>0.05$) based on one-way ANOVA and Tukey's multiple comparison post-test analyses.

FIGURE 4 depicts the threshold cycle of differing HPV16L1 primer concentrations. Both the sense and antisense primers were tested in combination at differing concentrations using 10 copies of HPV16L1 plasmid as the DNA template.

FIGURE 5 shows the ΔRn of differing HPV16L1 primer concentrations. Both the sense and antisense primers were tested in combination at differing concentrations using 10 copies of HPV16L1 plasmid as the DNA template.

FIGURE 6 shows the sensitivity of the HPV6 multiplex PCR assay. Results (mean ± SD, n=3) obtained with each specific probe are depicted by different

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to an assay for detection of HPV subtypes in a clinical sample that substantially reduces the risk of false negative results as compared to other assays known in the art.

It is well known that the relationship between the HPV genome and chromosomal host DNA may change during the multistage tumorigenic process (For review, see McMurray et al., *Int. J. Exp. Path.* 82: 15-33 (2001)). Premalignant lesions are often associated with episomal forms of HPV DNA while later-stage tumors typically have integrated HPV sequences. As a result of the integration correlated with advanced stages of disease progression, the open reading frame of specific HPV genes, such as the L1 locus, may become disrupted. Such disruption of HPV gene sequence may lead to false negative results in assays designed to specifically detect the disrupted sequence.

Therefore, a preferred embodiment of the present invention provides a method for identifying the presence of a specific HPV subtype in a sample, wherein said method comprises simultaneously detecting and amplifying a plurality of HPV genes of a single HPV subtype. A sample is considered positive for the HPV subtype if a majority of the plurality of the HPV genes are detected by the methods of the present invention. Another preferred embodiment of the present invention provides an assay for the presence of a specific HPV subtype, wherein said assay comprises simultaneously detecting and amplifying three HPV genes of a single HPV subtype. A sample is considered positive for the HPV subtype if at least two of the three genes are detected and HPV negative if none of the three genes are detected by the methods of the present invention. Said assay reduces the risk of obtaining false negative results associated with assays that test for a single HPV locus. The method of the present invention is highly specific and reproducible.

The method of the present invention for detecting HPV subtypes in a clinical sample also substantially reduces the risk of false positive results as compared to other assays known in the art. Such false positive results are caused by the high degree of homology among specific HPV genes as compared to the same HPV genes from a different HPV subtype. This level of homology makes it difficult to design a PCR assay that is specific for a single HPV subtype. When utilizing other methods known in the art that detect single loci, therefore, it is necessary to confirm positive results by serially testing for the presence of several loci of a single HPV-type. The further experimentation required to verify positive results is cumbersome and time-

sensitivity of the HPV Multiplex PCR assays for HPV6 (Fig. 10), HPV11 (Fig. 11), HPV16 (Fig. 12), and HPV18 (Fig. 13) was also confirmed using viral DNA isolated from human clinical samples.

Tremendous assay sensitivity, as exhibited by the methods of the present invention, is critical in screening clinical samples where the copy number of HPV may be low. Because the physical manifestations of HPV infection are often covert and the latency period prolonged, infection with HPV may not be detected until the patient has been diagnosed with cervical intraepithelial neoplasia (CIN), which, if allowed to go untreated, can progress to carcinoma. Typically, higher grade lesions (CIN2, CIN3 and carcinoma) are associated with high HPV copy number, which may be detectable by traditional methods known in the art. However, many assays currently in use are not sensitive or specific enough to detect low copy number HPV. Tremendous sensitivity is critical, therefore, for early detection of HPV when HPV copy numbers are low and therapeutic intervention is more likely to be effective.

The present invention more specifically relates to a method for detecting the presence of a human papillomavirus (HPV) subtype in a nucleic acid-containing sample comprising:

amplifying the nucleic acid in the presence of a nucleic acid polymerase and a plurality of oligonucleotide sets to produce a plurality of PCR amplicons;

wherein each oligonucleotide set consists of (a) a forward discriminatory PCR primer hybridizing to a first location of an HPV subtype, (b) a reverse discriminatory PCR primer hybridizing to a second location of the HPV subtype downstream of the first location, and (c) a fluorescent probe labeled with a quencher molecule and a fluorophore which emits energy at a unique emission maxima; said probe hybridizing to a location of the HPV subtype between the first and the second locations;

wherein each oligonucleotide set specifically hybridizes to a different HPV amplicon derived from the same HPV subtype;

allowing said nucleic acid polymerase to digest each fluorescent probe during amplification to dissociate said fluorophore from said quencher molecule;

detecting a change of fluorescence upon dissociation of the fluorophore and the quencher, the change of fluorescence corresponding to the occurrence of nucleic acid amplification; and

on wavelength) into a predictably spaced pattern across a charged-coupled device (CCD) camera. A Sequence Detection System application of the ABI PRISM® 7700 collects the fluorescent signals from the CCD camera and applies data analysis algorithms.

Nucleic acid polymerases for use in the methods of the present invention must possess 5' – 3' exonuclease activity. Several suitable polymerases are known in the art, for example, Taq (*Thermus aquaticus*), Tbr (*Thermus brockianus*) and Tth (*Thermus thermophilus*) polymerases. TAQ DNA polymerase is the preferred polymerase of the present invention. The 5' – 3' exonuclease activity is characterized by the degradation of double-stranded DNA encountered during extension of the PCR primer. A fluorescent probe annealed to the amplicon will be degraded in a similar manner, thus releasing the fluorophore from the oligonucleotide. Upon dissociation of the fluorophore and the quencher, the fluorescence emitted by the fluorophore is no longer quenched, which results in a detectable change in fluorescence. During exponential growth of the PCR product, the amplicon-specific fluorescence increases to a point at which the sequence detection application, after applying a multicomponenting algorithm to the composite spectrum, can distinguish it from the background fluorescence of non-amplifying samples. The ABI PRISM® 7700 Sequence Detection Instrument also comprises a software application, which determines the threshold cycle (Ct) for the samples (cycle at which this fluorescence increases above a pre-determined threshold). PCR negative samples have a Ct equal to the total number of cycles performed and PCR positive samples have a Ct less than the total number of cycles performed.

The present invention relates to a method for detecting the presence of a human papillomavirus (HPV) subtype in a nucleic acid-containing sample. In a preferred embodiment of this invention, the HPV subtype is selected from the group consisting of: HPV6, HPV11, HPV16 and HPV18.

In another preferred embodiment of the method of the present invention, the number of oligonucleotide sets is odd and the sample is positive for the HPV subtype tested if a change of fluorescence is detected in a majority of fluorophores.

In yet another preferred embodiment of the method of the present invention to identify the presence of a specific HPV subtype in a sample, the number of oligonucleotide sets is three. A sample is determined to be positive for the HPV subtype if a change of fluorescence is detected in at least two fluorophores. Samples

nucleic acid polymerase is AmpliTaq Gold DNA polymerase and the PCR cycling conditions include a "hot start" PCR reaction. Said polymerase is inactive until activation, which can be accomplished by incubating the PCR reaction components at 95°C for approximately 10 minutes prior to PCR cycling. PCR methods comprising a similar initial incubation step are known in the art as "hot start" PCR assays.

Preferably, oligonucleotide probes of the present invention are in the range of about 20 to about 40 nucleotides in length. More preferably, the oligonucleotide probe is in the range of about 18 to about 30 nucleotides in length. Most preferably, the oligonucleotide probe is in the range of about 24 to about 30 nucleotides in length. The precise sequence and length of an oligonucleotide probe of the invention depends in part on the nature of the target polynucleotide to which it binds. The binding location and length may be varied to achieve appropriate annealing and melting properties for a particular embodiment.

Preferably, the 3' terminal nucleotide of the oligonucleotide probe is blocked or rendered incapable of extension by a nucleic acid polymerase. Such blocking is conveniently carried out by phosphorylation of the 3' terminal nucleotide, since the DNA polymerase can only add nucleotides to a 3' hydroxyl and not a 3' phosphate.

It is preferred that HPV primers and probes of the present invention do not share full homology with other HPV subtypes. Each primer of the present invention should be designed so that 3' homology is lacking in at least one nucleotide or more. Such primer design would substantially reduce the chance of the primer annealing to the wrong HPV subtype and prevent primer extension if annealing to an HPV type that was not intended does occur since TAQ DNA Polymerase only extends a primer from the 3' end and requires that the 3' end be properly annealed.

It is also preferred that each probe contain mismatches along the length of the oligonucleotide which destabilize the oligonucleotide binding to non-specific HPV targets. As few as one mismatch along the length of the oligonucleotide probe is enough to discriminate between loci. Because the probe of the present invention is only hydrolized and detected when bound to the segment of DNA that is being amplified, non-specific binding of the probe to a DNA sequenced that is not being amplified is not detected.

To this end, the present invention relates to a primer pair for the PCR amplification of HPV nucleic acid, wherein both the forward and reverse PCR primers are discriminatory. In a preferred embodiment of the invention, the

the probe was designed to detect. In a preferred embodiment of this invention, the fluorophore is attached to a 5' terminal nucleotide of the specific sequence of nucleotides and the quencher is attached to a 3' terminal nucleotide of the specific sequence of nucleotides.

Preferably, fluorophores are fluorescent organic dyes derivatized for attachment to the 3' carbon or terminal 5' carbon of the probe via a linking moiety. Preferably, quencher molecules are also organic dyes, which may or may not be fluorescent, depending on the embodiment of the invention. For example, in a preferred embodiment of the invention, the quencher molecule is non-fluorescent. Generally, whether the quencher molecule is fluorescent or simply releases the transferred energy from the reporter by non-radiative decay, the absorption band of the quencher should substantially overlap the fluorescent emission band of the reporter molecule. Non-fluorescent quencher molecules that absorb energy from excited reporter molecules, but which do not release the energy radiatively, are referred to herein as "dark quenchers," "dark quencher molecules," "non-fluorescent quenchers" or "non-fluorescent quencher molecules".

Several fluorophore-quencher pairs are described in the art. See, e.g. Pesce et al, editors, *Fluorescence Spectroscopy*, Marcel Dekker, New York, (1971); White et al, *Fluorescence Analysis: A Practical Approach*, Marcel Dekker, New York, (1970); and the like. The literature also includes references providing exhaustive lists of fluorescent and non-fluorescent molecules and their relevant optical properties, e.g. Berlman, *Handbook of Fluorescence Sprectra of Aromatic Molecules*, 2nd Edition, Academic Press, New York, (1971). Further, there is extensive guidance in the literature for derivatizing reporter and quencher molecules for covalent attachment via common reactive groups that can be added to an oligonucleotide. See, e.g. U.S. Pat. No. 3,996, 345; and U.S. Pat. No. 4,351,760.

Exemplary fluorophore-quencher pairs may be selected from xanthene dyes, including fluoresceins, and rhodamine dyes. Many suitable forms of these compounds are widely available commercially with substituents on their phenyl moieties which can be used as the site for bonding or as the bonding functionality for attachment to an oligonucleotide. Another group of fluorescent compounds are the naphthylamines, having an amino group in the alpha or beta position. Included among such naphthylamino compounds are 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8- naphthalene sulfonate and 2-p-touidinyl-6-naphthalene sulfonate. Other dyes include 3-phenyl-7-isocyanatocoumarin, acridines, such as 9- isothiocyanatoacridine

with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

allowing said nucleic acid polymerase to digest each probe during amplification to dissociate said fluorophore from said quencher molecule;

detecting a change of fluorescence upon dissociation of the fluorophore and the quencher, the change of fluorescence corresponding to the occurrence of nucleic acid amplification; and

determining that the sample is positive for the HPV6 subtype if a change of fluorescence is detected with at least two probes.

In a preferred embodiment of the method for detecting the presence of HPV6 in a sample described above, the fluorophore of the first oligonucleotide set is FAM, the fluorophore of the second oligonucleotide set is JOE and the fluorophore of the third oligonucleotide set is TET.

The present invention further relates to a method for detecting the presence of HPV11 in a nucleic acid-containing sample comprising:

amplifying the nucleic acid in the presence of a nucleic acid polymerase and three oligonucleotide sets;

the first oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:10, a reverse discriminatory PCR primer as set forth in SEQ ID NO:11, and a probe as set forth in SEQ ID NO:12, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

the second oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:13, a reverse discriminatory PCR primer as set forth in SEQ ID NO:14, and a probe as set forth in SEQ ID NO:15, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

the third oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:16, a reverse discriminatory PCR primer as set forth in SEQ ID NO:17, and a probe as set forth in SEQ ID NO:18, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

allowing said nucleic acid polymerase to digest each probe during amplification to dissociate said fluorophore from said quencher molecule;

In a preferred embodiment of the method to detect the presence of HPV16 in a sample described above, the fluorophore of the first oligonucleotide set is FAM, the fluorophore of the second oligonucleotide set is JOE and the fluorophore of the third oligonucleotide set TET.

This invention additionally relates to a method for detecting the presence of HPV18 in a nucleic acid-containing sample comprising:

amplifying the nucleic acid in the presence of a nucleic acid polymerase and three oligonucleotide sets;

the first oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:28, a reverse discriminatory PCR primer as set forth in SEQ ID NO:29, and a probe as set forth in SEQ ID NO:30, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET

the second oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:31, a reverse discriminatory PCR primer as set forth in SEQ ID NO:32, and a probe as set forth in SEQ ID NO:33, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

the third oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:34, a reverse discriminatory PCR primer as set forth in SEQ ID NO:35, and a probe as set forth in SEQ ID NO:36, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

allowing said nucleic acid polymerase to digest each probe during amplification to dissociate said fluorophore from said quencher molecule;

detecting a change of fluorescence upon dissociation of the fluorophore and the quencher, the change of fluorescence corresponding to the occurrence of nucleic acid amplification; and

determining that the sample is positive for the HPV18 subtype if a change of fluorescence is detected with at least two probes.

In a preferred embodiment of the method to detect the presence of HPV18 in a sample described above, the fluorophore of the first oligonucleotide set is FAM, the fluorophore of the second oligonucleotide set is JOE and the fluorophore of the third oligonucleotide set is TET.

nucleotide, no guanosine residue on the 5' terminus and more cytosine residues than guanosine residues (see FIGURE 1).

The predicted cross-reactivity of each primer and probe to other known HPV subtypes was assessed by BLAST searching each sequence against the NCBI Genbank database. Most primer and probe sequences returned unique hits for the specific HPV for which they were designed and did not share any homology with other HPV subtypes. The HPV6L1 antisense primer shares some homology with HPV3, HPV26, HPV28 and HPV70. The HPV6L1 TaqMan probe shares some homology with HPV45, HPV54, HPV59, HPV66 and HPV83. The HPV6E6 antisense primer shares homology with HPV11. The HPV6E7 antisense primer shares some homology with HPV2. The HPV6E7 TaqMan probe shares some homology with HPV11, HPV42, HPV44, HPV55 and HPV74. The HPV11L1 sense primer shares some homology with HPV6. The HPV11L1 TaqMan probe shares some homology with HPVAE8, HPV6, HPV27, HPV31, HPV34, HPV55, HPV64, and HPV71. The HPV11E6 antisense primer shares some homology with HPV57. The HPV11E6 TaqMan probe shares some homology with HPV6. The HPV11E7 sense primer shares some homology with HPV6, HPV13, HPV44, HPV55, and HPV74. The HPV11E7 TaqMan probe shares some homology with HPV6 and HPV13. The HPV16L1 sense primer shares some homology with HPV68. The HPV16L1 antisense primer shares some homology with HPV35, HPV35H, HPV42 and HPV52. the HPV16L1 TaqMan probe shares some homology with HPV7, HPV10, HPV35, and HPV35H. The HPV18E6 antisense primer shares some homology with HPV85. The HPV18E7 sense primer shares some homology with HPV6, HPV39 and HPV39. The HPV18E7 TaqMan probe shares some homology with HPV59.

None of the HPV primers and probes that were designed share full homology with other HPV subtypes. Each primer lacks 3' homology of at least one nucleotide or more which suggests that even if it were to anneal to the wrong HPV subtype, it would not be extended since TAQ DNA Polymerase only extends a primer from the 3' end and requires that the 3' end be properly annealed. Each TaqMan probe contains mismatches along the length of the oligonucleotide which destabilize the oligonucleotide binding to non-specific targets. As few as one mismatch along the length of the oligonucleotide probe is enough to discriminate between loci. In addition, the probe is only hydrolized and detected when bound to the segment of

City, CA), 200 nM final concentration of each primer, 100 copies of plasmid DNA template, DEPC-treated water (Ambion) and a range of concentrations (25-200 nM) of fluorescently-labeled oligonucleotide probes. The cycling conditions consisted of an initial step of 50°C for 2 min followed by 95°C for 10 min, and 45 cycles of 94°C for 15 sec and 60°C for 1 min.

Included in the Taq-Man Universal PCR master mix is dUTP (instead of dTTP) and uracil-N-glycosylase (UNG), an enzyme that is activated at 50°C and cleaves uracil-containing nucleic acids. See Longo et al., *Gene* 93: 125-128 (1990). UNG prevents the reamplification of carryover PCR products in subsequent experiments.

A concentration of each probe was selected that exhibited the lowest Ct (for example HPV16L1; FIGURE 2 and a ΔRn ~ 1 (for example HPV16L1; FIGURE 3). The primer concentrations were optimized for each locus by assessing the Ct and ΔRn of each primer concentration combination in a fine matrix assay using the previously determined concentration of loci-specific oligonucleotide probe and ten copies of the plasmid DNA template. The concentrations of the sense and antisense primers that exhibited the lowest Ct (for example HPV16L1; FIGURE 4) and maximal ΔRn (for example HPV16L1; FIGURE 5) were selected.

The primers and probes were then tested together with the addition of extra AmpliTaq Gold DNA Polymerase (0.75 U/well, Applied Biosystems, Foster City, CA). The additional DNA polymerase was added because the TaqMan Universal 2X PCR Master Mix, which already contains AmpliTaq Gold DNA Polymerase, was optimized for duplex reactions and not for triplex reactions. The additional DNA polymerase supplements the DNA polymerase in the 2X master mix and reinforces the reaction.

The linearity and sensitivity of each PCR assay was confirmed using loci-specific plasmids at concentrations ranging from 10 to 10^6 copies/reaction. The HPV6 (FIGURE 6), HPV11 (FIGURE 7), HPV16 (FIGURE 8) and HPV18 (FIGURE 9) multiplex PCR assays were linear within the range of 10 to 10^6 copies. The sensitivity of the HPV multiplex PCR assays for HPV6 (FIGURE 10), HPV11 (FIGURE 11), HPV16 (FIGURE 12), and HPV18 (FIGURE 13) was also confirmed using serially diluted HPV viral DNA isolated from human clinical specimens (see EXAMPLE 5).

based on the threshold cycle of each sample. Data from all three dye layers were then compiled by the workbook, which calculates a consensus HPV PCR positivity of each sample based on the rules set above.

FIGURES 10-13 show the sensitivity of the HPV multiplex PCR assays for HPV6, HPV11, HPV16, and HPV18, respectively, using serially diluted HPV viral DNA isolated from human clinical specimens. High concentration viral DNA from clinical samples was diluted 10-fold serially in a background of human genomic DNA and run in the appropriate multiplex PCR assay. After a 100,000 to 1,000,000-fold dilution (approximate copy number 1-10), viral DNA was still detectable. Viral detection at low copy numbers was linear over the entire dilution range.

5. The method of claim 1, wherein the quencher is non-fluorescent.

6. The method of claim 3, wherein the fluorophores are FAM, JOE and TET and the quencher is BHQ1.

7. The method of claim 1, wherein the HPV subtype is selected from the group consisting of: HPV6, HPV11, HPV16 and HPV18.

8. The method of claim 3, wherein the HPV subtype is selected from the group consisting of: HPV6, HPV11, HPV16 and HPV18.

9. An oligonucleotide probe comprising a sequence of nucleotides selected from the group consisting of: SEQ ID NO:3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:18, SEQ ID NO:21, SEQ ID NO:24, SEQ ID NO:27, SEQ ID NO: 30, SEQ ID NO: 33 and SEQ ID NO:36.

10. The oligonucleotide probe of claim 9 further comprising a fluorophore and a quencher molecule.

11. The oligonucleotide probe of claim 10, wherein the fluorophore is attached to a 5' terminal nucleotide of the sequence of nucleotides and the quencher is attached to a 3' terminal nucleotide of the sequence of nucleotides.

12. The oligonucleotide probe of claim 10, wherein the fluorophore is selected from the group consisting of: FAM, JOE and TET.

13. The oligonucleotide probe of claim 10, wherein the quencher molecule is non-fluorescent.

14. The oligonucleotide probe of claim 13, wherein the quencher molecule is BHQ1.

(b) allowing said nucleic acid polymerase to digest each probe during amplification to dissociate said fluorophore from said quencher molecule;

(c) detecting a change of fluorescence upon dissociation of the fluorophore and the quencher, the change of fluorescence corresponding to the occurrence of nucleic acid amplification; and

(d) determining that the sample is positive for the HPV6 subtype if a change of fluorescence is detected with at least two probes.

17. The method of claim 16 wherein the fluorophore of the first oligonucleotide set is FAM, the fluorophore of the second oligonucleotide set is JOE and the fluorophore of the third oligonucleotide set is TET.

18. A method for detecting the presence of HPV11 in a nucleic acid-containing sample comprising:

(a) amplifying the nucleic acid in the presence of a nucleic acid polymerase and three oligonucleotide sets;

the first oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:10, a reverse discriminatory PCR primer as set forth in SEQ ID NO:11, and a probe as set forth in SEQ ID NO:12, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

the second oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:13, a reverse discriminatory PCR primer as set forth in SEQ ID NO:14, and a probe as set forth in SEQ ID NO:15, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

the third oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:16, a reverse discriminatory PCR primer as set forth in SEQ ID NO:17, and a probe as set forth in SEQ ID NO:18, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

(b) allowing said nucleic acid polymerase to digest each probe during amplification to dissociate said fluorophore from said quencher molecule;

(d) determining that the sample is positive for the HPV16 subtype if a change of fluorescence is detected with at least two probes.

21. The method of claim 20, wherein the fluorophore of the first oligonucleotide set is FAM, the fluorophore of the second oligonucleotide set is JOE and the fluorophore of the third oligonucleotide set TET.

22. A method for detecting the presence of HPV18 in a nucleic acid-containing sample comprising:

(a) amplifying the nucleic acid in the presence of a nucleic acid polymerase and three oligonucleotide sets;

the first oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:28, a reverse discriminatory PCR primer as set forth in SEQ ID NO:29, and a probe as set forth in SEQ ID NO:30, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET

the second oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:31, a reverse discriminatory PCR primer as set forth in SEQ ID NO:32, and a probe as set forth in SEQ ID NO:33, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

the third oligonucleotide set consisting of a forward discriminatory PCR primer as set forth in SEQ ID NO:34, a reverse discriminatory PCR primer as set forth in SEQ ID NO:35, and a probe as set forth in SEQ ID NO:36, said probe labeled with BHQ1 on the 3' end and a fluorophore on the 5' end, said fluorophore selected from the group consisting of FAM, JOE and TET;

(b) allowing said nucleic acid polymerase to digest each probe during amplification to dissociate said fluorophore from said quencher molecule;

(c) detecting a change of fluorescence upon dissociation of the fluorophore and the quencher, the change of fluorescence corresponding to the occurrence of nucleic acid amplification; and

(d) determining that the sample is positive for the HPV18 subtype if a change of fluorescence is detected with at least two probes.

Figure 1 (page 1 of 2)
Primer and Probe Sequences for HPV Multiplex PCR assays

Oligo Name	Sequence	Position	Final Conc
HPV6L1 Sense (SEQ ID NO:1)	5'-GACTCGTCTCTTTTTGATCCCACA-3'	250-273	125 nM
HPV6L1 Antisense (SEQ ID NO: 2)	5'-TAGGAAAGGATGTCCACTTACACCC-3'	336-360	125 nM
HPV6LI Probe (SEQ ID NO: 3)	5'-CAACGTTTGGTATGGGCATGCACAGGCCTA-3'	277-306	100 nM
HPV6E6 Sense (SEQ ID NO: 4)	5'-TAAAGGTCCTGTTTCGAGGCG-3'	152-172	150 n M
HPV6E6 Antisense (SEQ ID NO: 5)	5'-TGACACAGGTAGCACCGAATTAG-3'	301-323	150 nM
HPV6E6 Probe (SEQ ID NO: 6)	5'-ATCCATATGCAGCCTGCGCGTGCTGCC-3'	176-202	125 nM
HPV6E7 Sense (SEQ ID NO: 7)	5'-ACGAAGTGGACGGACAAGATTC-3'	113-134	125 nM
HPV6E7 Antisense (SEQ ID NO: 8)	5'-TCCCAACAGAAGCTGTTGCACT-3'	234-255	100 nM
HPV6E7 Probe (SEQ ID NO: 9)	5'-CTGTACACTGCACAACCAGTCGAACGTTGC-3'	188-217	75 n M
HPV11L1 Sense (SEQ ID NO: 10)	5'-CCTCCACCAAATGGTACACTGGAG-3'	1210-1233	400 nM
HPV11L1 Antisense (SEQ ID NO: 11)	5'-CCGTCCTCGATATCCACTTTGC-3'	1398-1419	400 nM
HPV11LI Probe (SEQ ID NO: 12)	5'-CAGTCACAGGCCATTACCTGTCAGAAACC-3'	1252-1280	75 nM
HPV11E6 Sense (SEQ ID NO: 13)	5'-TTTGCACACTCTGCAAATTCAGTGC-3'	69-93	200 nM
HPV11E6 Antisense (SEQ ID NO: 14)	5'-TTGCAGTTCTAAGCAACAGGCACACG-3'	188-213	200 nM
HPV11E6 Probe (SEQ ID NO: 15)	5'- CATATATCTCTGCGGTGGTCAGTGCATTCC -3	104-133	150 nM
HPV11E7 Sense (SEQ ID NO: 16)	5'-AGCTCAGAAGATGAGGTGGACAAG -3'	94-117	100 nM
HPV11E7 Antisense (SEQ ID NO: 17)	5'-TGCCCAGCAAAAGGTCTTGTAG -3'	235-256	200 nM
HPV11E7 Probe (SEQ ID NO: 18)	5'- CACTCCACAACCAGTCGGACGTTGCT-3'	187-212	75 nM

Figure 2
HPV16L1-FAM TaqMan dual-labeled probe concentration curve

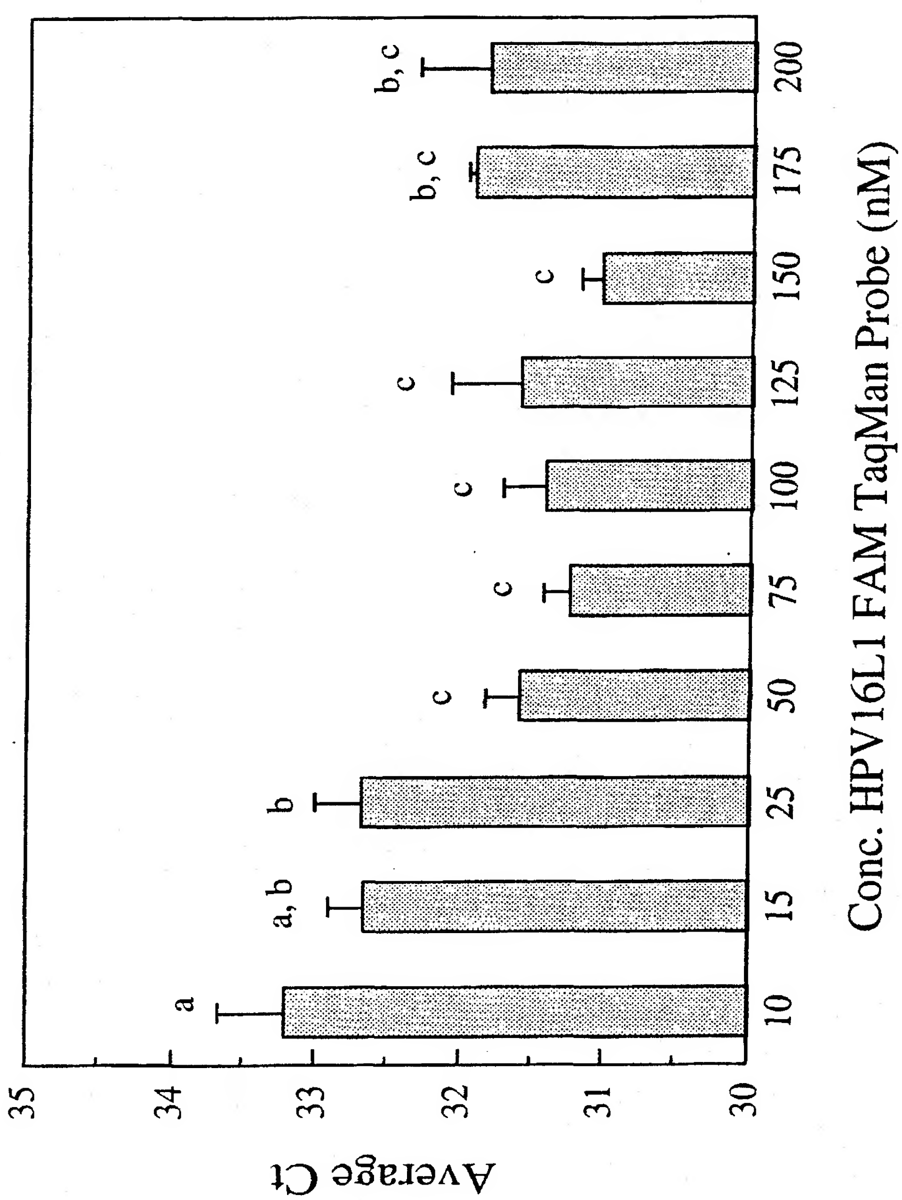


Figure 4
Threshold cycle of differing HPV16L1 primer concentrations

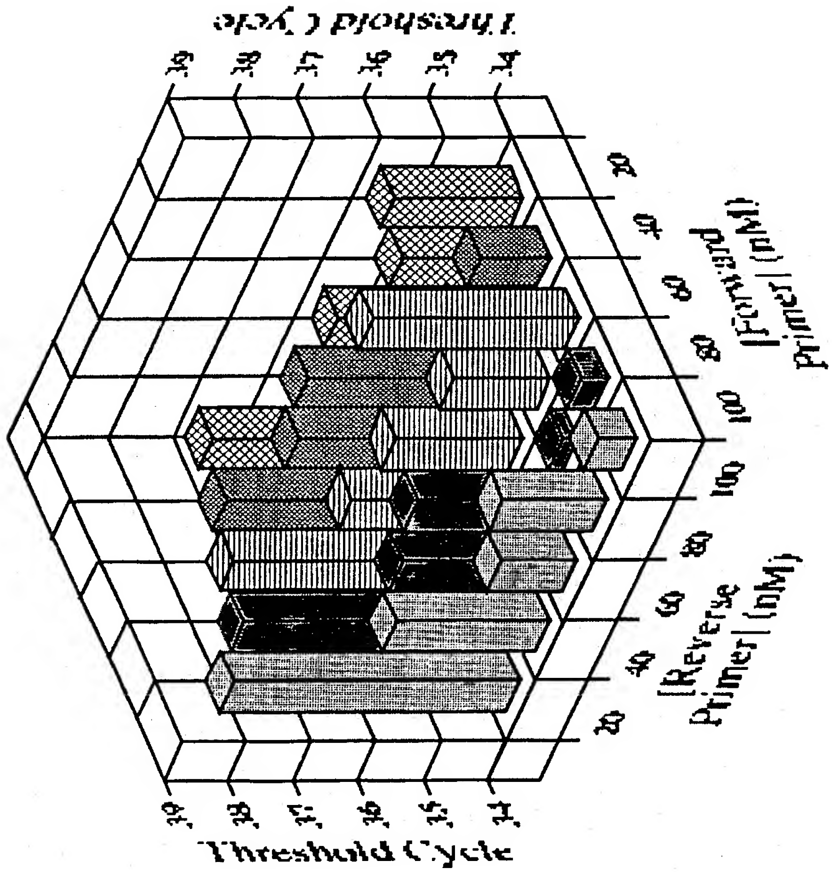


Figure 6

Sensitivity of the HPV6 Multiplex PCR Assay –Plasmid DNA

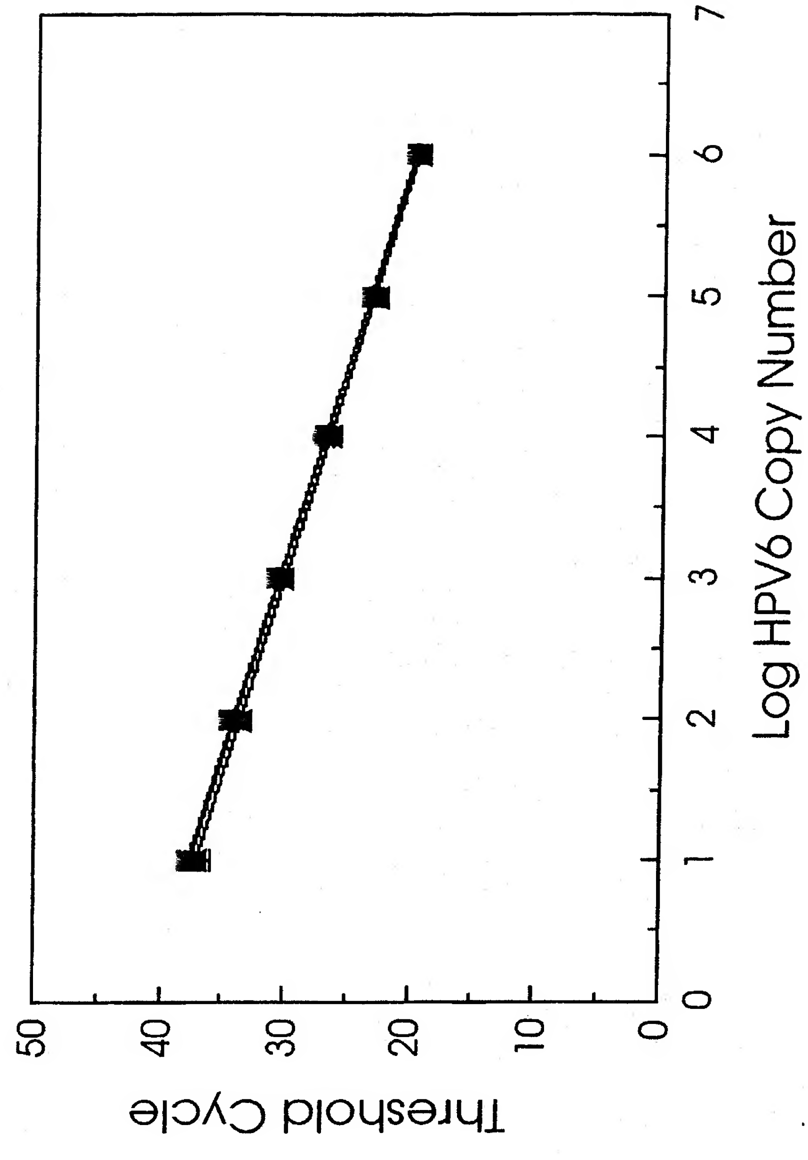


Figure 8

Sensitivity of the HPV16 Multiplex PCR Assay – Plasmid DNA

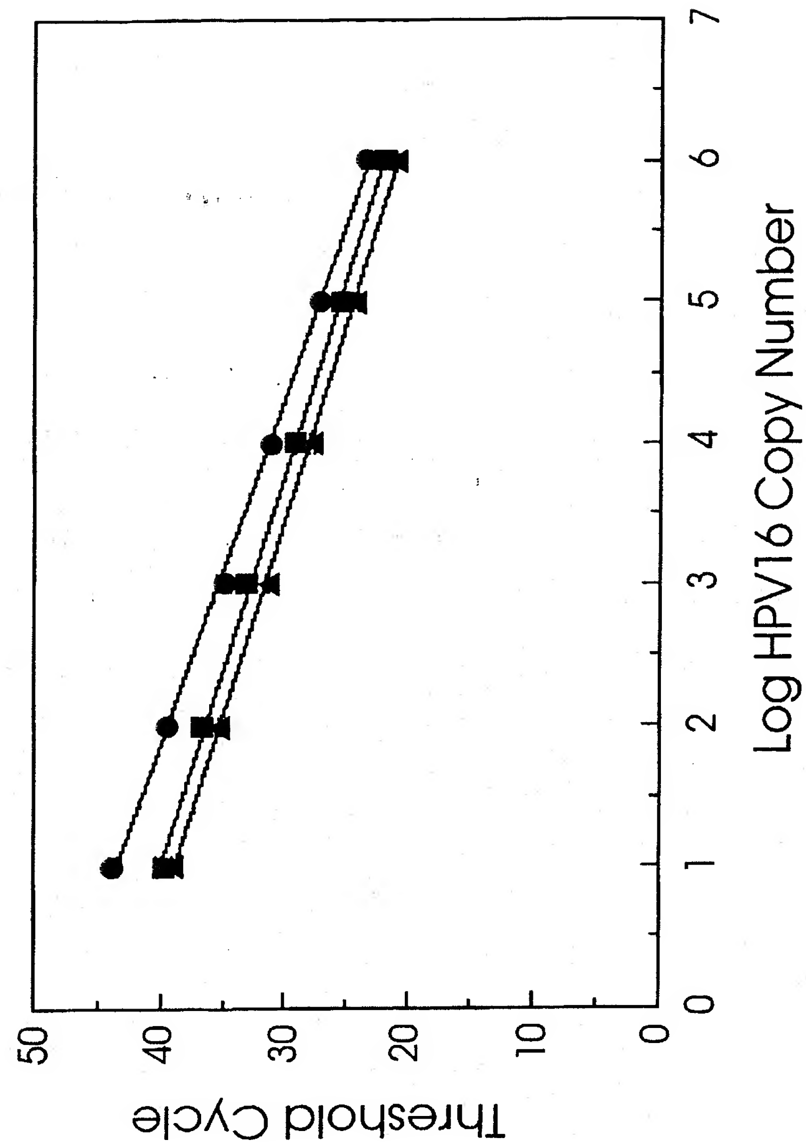


Figure 10
Sensitivity of the HPV6 Multiplex PCR Assay – Viral DNA

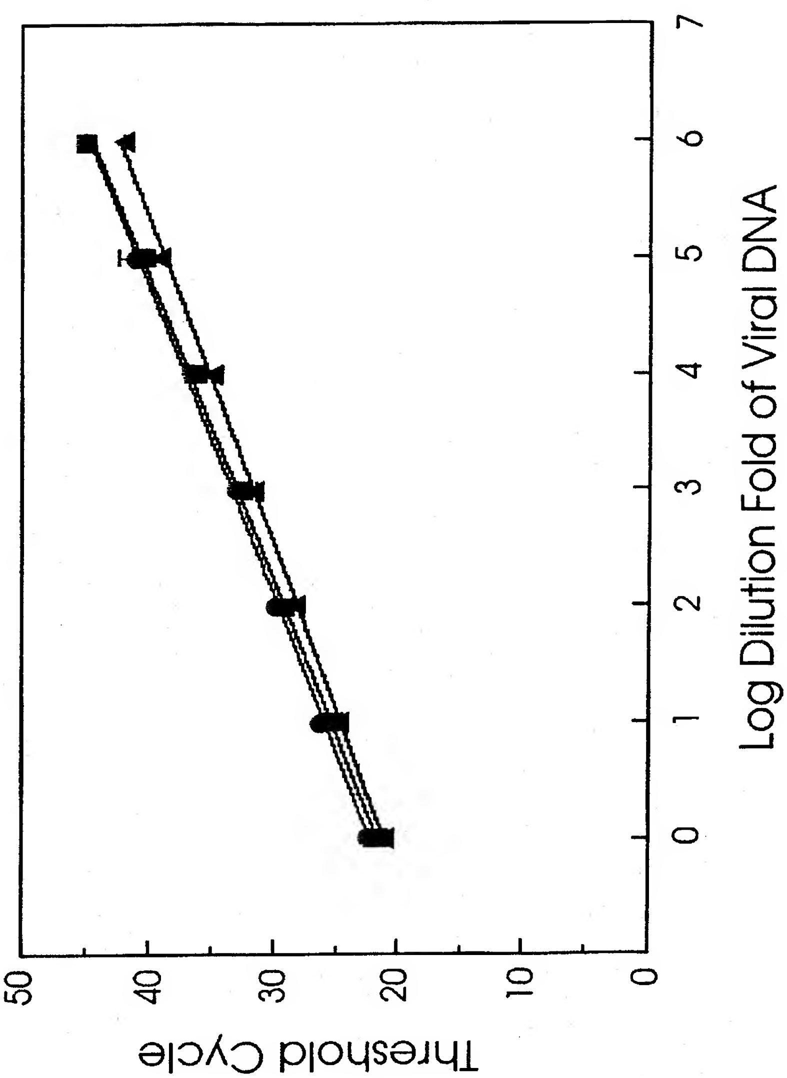
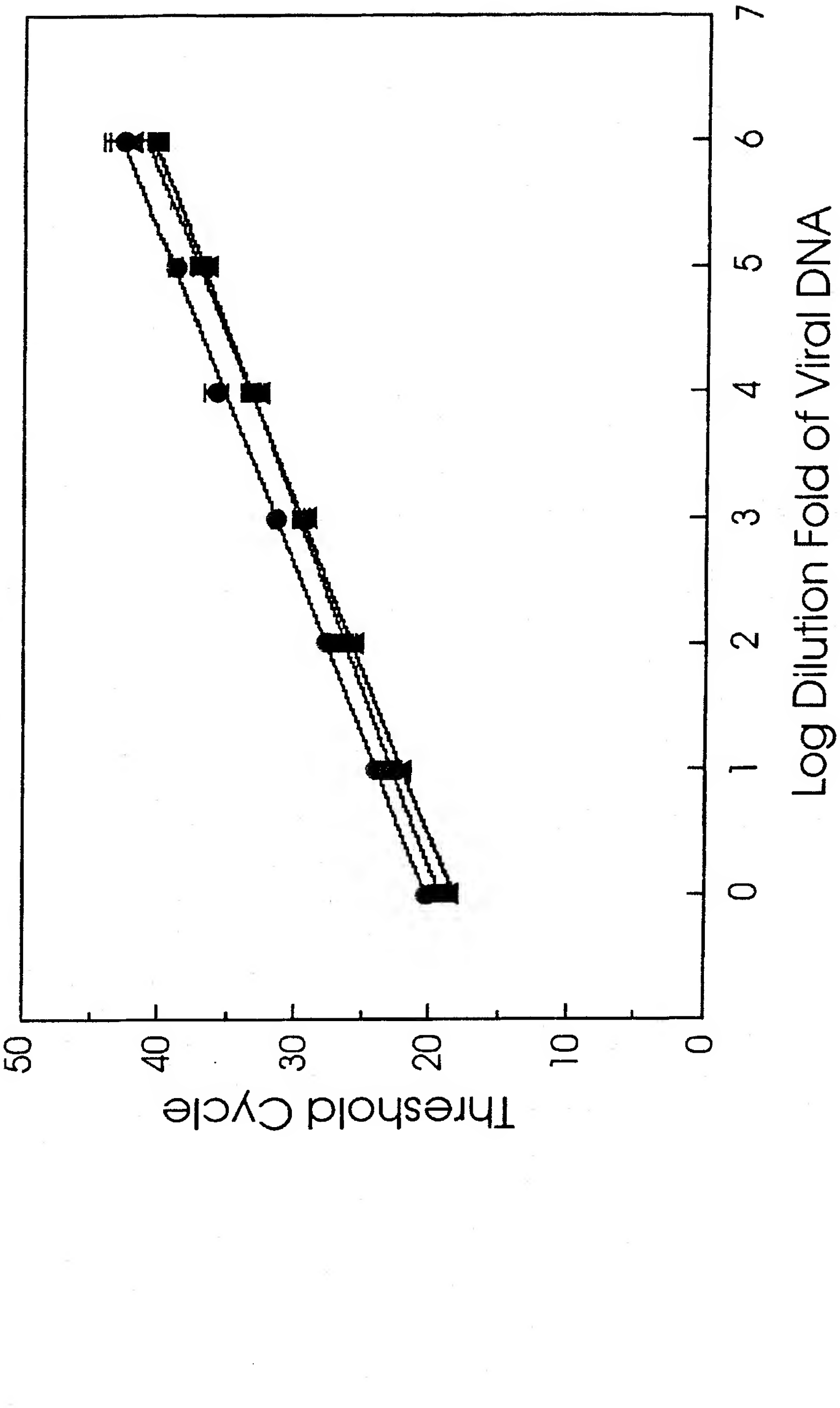


Figure 12
Sensitivity of the HPV16 Multiplex PCR Assay – Viral DNA



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Jansen, Kathrin
Taddeo, Frank J.
Li, Weili
DiCello, Anthony C.

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